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D-81245 München (DE)(54) **Gene coding for a polypeptide which enhances virus infection of host insects.**

(57) Isolated and cloned baculovirus genes encoding a polypeptide protein present in the occlusion body of certain baculoviruses such as Trichoplusia ni granulosis virus and Pseudaletia unipuncta granulosis virus Hawaiian strain, said genes possessing a biological activity of enhancing virus infection of host insects by causing rapid degradation of the peritrophic membrane lining the midgut lumen of insects. The invention is also directed to pesticides incorporating said genes, i.e. the purified conserved and enhancing baculovirus proteins herein termed enhancins.

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BACKGROUND OF THE INVENTION

The invention relates to the cloning and sequencing of novel viral genes from certain baculoviruses for insect control. More particularly, the invention relates to an isolated and cloned DNA from a granulosis virus which comprises an amino acid sequence of the viral gene encoding a polypeptide isolated from occlusion bodies of certain baculoviruses and which polypeptide possesses the biological activity of enhancing baculovirus infectivity. This invention also relates to isolated and purified baculovirus proteins which are characterized by enhancing the infectivity of baculoviruses. Such proteins termed herein as "enhancins" are found within the viral occlusion body, have a disruptive effect on the insect peritrophic membrane (PM) proteins, and/or interact with the midgut epithelium in such a manner as to permit the increased adsorption, penetration and uptake of virus particles by midgut cells with a concomitant increase in host mortality.

The publications used to illuminate the background of the invention, and in particular cases, to provide additional details respecting its practice are incorporated herein by reference, and for convenience, are numerically referenced by the following text and respectively grouped in the appended bibliography. Copies of all of the references mentioned in this bibliography are attached to the **INFORMATION DISCLOSURE STATEMENT** filed concurrently herewith.

FIELD OF THE INVENTION

Present in the protein occlusion bodies (OBs) of some baculoviruses is a unique viral-encoded protein which enhances viral infection of the host insect. This protein is referred to herein as the virus enhancing factor (VEF) and/or as the synergistic factor (SF). Pest control compositions comprising this factor and nuclear polyhedrosis viruses are the subject matter of U.S. Patent Nos. 4,973,667 and 5,011,685.

Studies on the mode of action of the VEF isolated from *Trichoplusia ni* (cabbage looper) granulosis virus (TnGV) showed that the VEF caused rapid degradation of the peritrophic membrane which lines the midgut lumen of lepidopterous larvae. Larval bioassays suggested that this alteration made the peritrophic membrane more permeable to invading baculoviruses resulting in at least a 25-fold increase in larval mortality (1,2).

DESCRIPTION OF RELATED ART

Closely related to, or identical with, the VEF protein is a lipoprotein, originally isolated in crude form from a Hawaiian strain of *Pseudaletia unipuncta* granulosis virus (PuGV-H), but not cloned or sequenced. It is described by Tanada and co-workers (7, 8, 10) as the "synergistic factor" (SF) and as having a calculated molecular weight between 90K and 160K (6, 24, 35, 42 and 43). The SF was released from the capsule upon dissolution in the midgut, and was localized to the microvillar surface of the midgut cell membrane (9,37) where it caused an apparent increase in the uptake of enveloped nucleocapsids (36). The binding of SF to the midgut membrane was found to be specific with a calculated equilibrium constant of 1.57×10^{-9} M (39).

It has been postulated by Hashimoto et al (23) that the two proteins (VEF and SF) are closely related and have similar dual modes-of-action: peritrophic membrane disruption and increased virus uptake. Evidence to support this relationship comes from southern hybridizations of PuGV-H genomic DNA with the VEF gene and western blots of dissolved PuGV-H occlusion bodies with an anti-VEF polyclonal antiserum (23). Tanada determined that this SF in the capsule of PuGV-H increased the larval susceptibility to *P. unipuncta* nuclear polyhedrosis virus (PuNPV) (8).

Since viral enhancing proteins are important at early stages of host infection, it is important to identify and locate the position of the VEF gene and the SF gene on the viral genome. A need, therefore, exists to clone and sequence both the VEF gene of TnGV and the SF gene of PuGV-H. It is an object of this invention to satisfy such a need. Another object is to compare the SF and TnGV VEF genes by showing their extremely high degree of sequence similarity and by demonstrating their similar effects on *T. ni* PMs and AcMNPV infections in *T. ni* larvae. Still another object is to show sequence homology and/or serological relatedness of the virulence genes and/or enhancing proteins among different baculoviruses.

SUMMARY OF THE INVENTION

The above-mentioned objects of the present invention, which will hereinafter become more readily apparent from the following description, have been attained by first isolating and purifying the VEF gene, which comprises a DNA molecule encoding a polypeptide of molecular weight 104 Kd and is found in the

granulin fraction of TnGV OBs purified by SEPHACRYL® S200 SUPERFINE (2.6 x 34 cm) column, possessing a biological activity and wherein said polypeptide has a total of 901 amino acid residues in the amino acid sequence of the polypeptide. Besides cloning and sequencing the gene encoding the viral enhancing factor (VEF) of TnGV, applicants have also successfully isolated the SF gene and determined its complete nucleotide sequence.

The gene encoding for the viral enhancing factor (VEF) of TnGV has been cloned from a lambda gIII expression library, and the complete nucleotide sequence determined. The VEF gene encodes a protein with a predicted molecular weight of 104 Kd which does not share homology to any previously reported proteins. The apparent promoter is located 4 bp upstream of the initiation codon and represents a consensus baculovirus late promoter (ATAAG). This has been confirmed by the identification of VEF mRNA in northern blots of infected larvae at 6 days but not 3 days post infection. Three repeats of the sequence 'TTACAAGA' which match the baculovirus late promoter in 4 of 5 nucleotide have been identified between 149 and 192 bp upstream of the initiation codon. While the function of these sequences is unknown, they are not believed to be transcriptionally active since they diverge from the consensus promoter at the invariant 'T' position. Using the VEF gene as a probe in southern blots of genomic DNAs, homologous sequences have been identified in PuGV-H and *Heliothis armigera* GV (HaGV) but not *Erinnyis ello* GV, (EeGV), *Autographa californica* nuclear polyhedrosis virus (AcMNPV) or *Trichoplusia ni* nuclear polyhedrosis virus (TnSNPV). In addition, SDS-PAGE analysis of dissolved viral occlusion bodies have demonstrated proteins with a molecular weight similar to VEF in PuGV-H and HaGV.

As pointed out above, the gene encoding the synergistic factor (SF) of PuGV-H has been cloned by applicants and the complete nucleotide sequence determined. The SF gene encodes a protein with a predicted molecular weight of 104Kd which shares a 99.1% and 98.2% homology with the nucleotide and amino acid sequence of the viral enhancing factor (VEF) gene of TnGV, respectively. A majority of the differences in the amino acid sequences of the two viruses result from two reciprocal frameshifts which occur between nucleotide +1962 and +1985 of the SF gene. Both enhancing proteins have similar activity in neonate larvae of *T. ni* (2.4 fold enhancement) and *in vitro* peritrophic membrane assays. Using a polyclonal antibody directed against TnGV VEF, 17 baculoviruses were screened by western blot hybridization. Cross reactive proteins are found in seven GVs isolated from 4 families of Lepidoptera. These putative enhancing proteins can be separated into 3 groups based on size: HaGV (110Kd); PuGV-H, *Pieris rapae* GV (PrGV), *Scotogramma trifolii* GV (StGV), and TnGV (104Kd); and *Cydia pomonella* GV (CpGV) and *Estigmene acrea* GV (80Kd). The name "enhancin" has been proposed for these enhancing proteins.

DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention and the attendant advantages thereof will be readily attained as the same becomes better understood by reference to the following details of description when considered in connection with the accompanying drawings, i.e. Figures 1-11.

Fig. 1. Mapping of the VEF gene of TnGV. a) a Hind III restriction map of the TnGV genome. By convention, the smallest fragment containing all of the granulin gene is assigned to be the first fragment at the left of the linearized map. A fine map of the b) Hind III-M fragment of TnGV and c) fusion gene of lambda F. The striped box indicates the position of the VEF gene while the open box indicates non-coding TnGV sequences inserted into the lambda gIII. The entire insert in lambda F is demarcated by the asterisks. The size of the DNA is indicated by scale, and the restriction sites for BamHI(B), ClaI(C), EcoRI(E), HindIII(H), KpnI(K), and SalI(S) are indicated.

Fig. 2. Western bolt analysis of lambda lysogens from lambda F (lane 1 or lambda gIII (lane 2) probed with either anti-VEF polyclonal antibody (lane 1) or an anti- β -galactosidase monoclonal antibody (lane 2). Lysogens were first separated on a 10% SDS-PAGE gel and then electrophoretically transferred to nitrocellulose. The 153Kd protein identified by the anti-VEF polyclonal antibody consists of 39Kd VEF carboxy-terminal and 114Kd of β -galactosidase.

Fig. 3. The nucleotide sequence of the VEF gene from TnGV. The gene has been translated using the single-letter amino acid code. The bolded sequence represents the consensus baculovirus late promoter (5), and the underlined sequences represents 3 repeats of the sequence (TTACAAGA) which matches the promoter in 4 of the 5 base pairs. Double underlined sequences indicate possible glycosylation sites. The DNA sequence of a 3.5 Kb portion of Hind III-M fragment was determined by dideoxy chain termination method using bacteriophage T7 DNA polymerase. Sequence data were compiled and analyzed using the software program of PCGENE. In this sequence, A stands for deoxyadenyl, G for deoxyguanyl, C deoxycytidyl, and T is thymidyl. The amino acids encoded by the above DNA are designated below the appropriate nucleotide triplet. Accordingly, M is methionine; K is lysine, P is proline; E is glutamate, L is

leucine; T is threonine; A is alanine; S is serine; V is valine; F is phenylalanine; I is isoleucine; G is glycine; D is aspartic acid; Q is glutamine; R is arginine; C is cysteine; W is tryptophan; N is asparagine; H is histidine; and Y is tyrosine.

Fig. 4. Northern blot of total RNA isolated from infected larvae. Total RNA was isolated from *T. ni* larvae at 3 and 6 days post inoculation (PI) with TnGV. Ten micrograms of RNA were electrophoresed in a denaturing 1.5% agarose and northern blotted following the methods of Dwyer and Granados (17). Blots were probed with the internal KpnI fragment of TnGV-VEF gene under high stringency conditions. No hybridization was found to RNA isolated at 3 days PI. However, 2 RNA species of 2.7 and 3.3 Kbp hybridized at 6 days PI. This indicated that the VEF gene was probably a late gene.

Fig. 5. Southern hybridization and SDS-PAGE analysis of TnGV and 5 other baculoviruses. a) Genomic baculovirus was digested with Hind III and electrophoresed on a 0.75% agarose gel. The DNA was transferred to nitrocellulose and probed with the internal KpnI fragment of TnGV-VEF and washed under high stringency conditions. Homologous sequences were identified in PuGV-H, and HaGV. b) Occlusion bodies are dissolved in 0.05 M NaCO₃ pH 10.5 for 15 minutes at room temperature and the nucleocapsids pelleted by centrifugation at 14,000 xg. The supernatants were removed and electrophoresed in a 10% SDS-PAGE gel and stained with COOMASSIE blue.

Fig. 6. A comparison of the nucleotide sequence for the PuGV-H SF and TnGV VEF genes. Hyphens denote nucleotide identical to the PuGV-H sequence. The consensus baculovirus late promoters (30) have been underscored, the putative start codon has been bolded, and the stop codon overscored. Two frameshift mutations have occurred at +1962 and +1985. The homology between the two genes is 99.1%. The second open reading frame starts at +2755 nt. The stop codon for this gene has not been found.

Fig. 7. A comparison of the amino acid sequence for the PuGV-H SF and TnGV VEF proteins. Hyphens denote nucleotide identical to the PuGV-H sequence. The identity between the two proteins is 98.2%.

Fig. 8. SDS-PAGE analysis of purified SF and VEF. Three micrograms of VEF or SF was added to an equal volume of 2X SDS-PAGE loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol). The sample were electrophoresed for 4.5 hours at 30 mAmps through a 7% separating gel (10 cm x 12 cm x 1.5 mm), and stained with COOMASSIE blue R-250 following standard protocols. SF (lane 1) migrated at 106K while VEF (lane 2) migrated at 101K.

Fig. 9. SDS-PAGE analysis of in vitro digest of peritrophic membranes. Individual peritrophic membranes (PM) were dissected from the last instar (a) *Trichoplusia ni* and (b) *Pseudaletia unipuncta* larvae. Peritrophic membranes were resuspended in 50 µl of 0.1 M Na₂CO₃ pH10.5 containing (a) 5 µg or (b) 10 µg of VEF (lane 2) or SF (lane 3), and incubated at 28° C for 60 min. The reactions were stopped by washing the PMs in deionized water and resuspending in SDS-PAGE loading buffer (0.062 M Tris-HCl pH 6.8, 2% SDS, 5% - mercaptoethanol). Controls (ln 1) consisted of PMs treated in the same manner but without enhancing protein. Samples were electrophoresed through at 10% separating gel and silver stained. SF and VEF digested the same protein in both the *T. ni* and *P. unipuncta* PMs. Multiple degradation products are evident in both the SF (ln 3) and VEF (ln 2) lanes.

Fig. 10. SDS-PAGE and Western blot analysis of nine baculoviruses. Viral occlusion bodies were dissolved in DAS (0.1 M Na₂CO₃, 0.01 M EDTA, 0.17 M NaCl, pH 10.9) for 15 min at room temperature. An equal volume of 2X SDS-PAGE loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) was added and the samples electrophoresed through a 10% separating gel. The gels were either (a) stained with COOMASSIE blue (b) or used in Western blots. Proteins cross-reacting with an anti-VEF/TrpE (23) antiserum were found in CpGV (lane 1, 80Kd), EaGV (Lane 2, 80Kd), HaGV (lane 4, 110Kd), PrGV (lane 7, 101Kd) and PuGV-H (lane 8, 106Kd). EeGV (lane 3), PbGV (lane 5), PiGV (lane 6), PuGV-O (lane 9) did not cross-react with the antiserum.

Fig. 11. SDS-PAGE and Western blot analysis of nine baculoviruses. Viral occlusion bodies were dissolved in DAS (0.1 M Na₂CO₃, 0.01 M EDTA, 0.17 M NaCl, pH 10.9) for 15 min at room temperature. An equal volume of 2X SDS-PAGE loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) was added and the samples electrophoresed through a 10% separating gel. The gels were either (a) stained with COOMASSIE blue (b) or used in Western blots. Proteins cross-reacting with an anti-VEF/TrpE (23) antiserum were found in StGV (lane 11, 106Kd), and TnGV (lane 12, 104Kd). SiGV (lane 10), AcMNPV (lane 13), AgMNPV (lane 14), CfNPV (lane 15), HzSNPV-ELCAR (lane 16), and TnSNPV (lane 17), did not cross-react with the antiserum.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTSEnhancing Protein Purification

The TnGV VEF and PuGV-H SF were isolated according to the methods of Gallo et al. (21) with the following modifications. Sephacryl S-200 was replaced by Sephacryl S-300 HR and the initial concentration of viral occlusion bodies was reduced from 1.7×10^{12} to 1.0×10^{12} per ml.

Purified enhancing factor containing approximately 3 mg of protein was added to an equal volume of 2X sample buffer (2X = 0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), heated in a boiling water bath for 3 min., and separated by SDS polyacrylamide gel electrophoresis (PAGE) according to the methods of Laemmli (29). Electrophoresis was carried out at 30 mAmps for 4.5 hr in a 7% separating gel (10 cm x 12 cm x 1.5 mm), and stained with COOMASSIE Blue R-250 following standard protocols.

Cloning and Sequencing of Enhancing Genes

The VEF present in granulin fraction of TnGV OBs was purified in the following manner:

1.7×10^{12} TnGV OBs were dissolved in 1 ml 0.05 M Na_2CO_3 for 15 min. at room temperature, and layered on a 20% sucrose cushion in H_2O and centrifuged for 45 min. at 126,000 g at 4°C. The granulin fraction remained on top of the sucrose cushion and was collected. After an incubation of 5 hrs at 28°C, the granulin fraction was applied onto a Sephacryl-S-200 column (2.6 x 34 cm) and eluted with 50 mM Tris-HCl pH 7.0, 0.1 M NaCl at 1.5 ml/min, and the absorption of the eluate measured at 280 nm. The first peak containing VEF protein was pooled and used for experiment.

A cloning and expression vector, lambda gt10, was used for construction of genomic library of TnGV and for isolation of the VEF gene (3). Antibodies were raised against Sephacryl-column purified VEF from granulin fraction after alkali solubilization of OBs (1) and were used for immunoblotting to screen for positive clones. Through several steps of screening approximately 6000 plaques, a clone was selected containing the longest viral-VEF DNA insert. Southern blot hybridization analysis of TnGV DNA Hind III digests, probed with the VEF clone insert, revealed that the VEF gene existed on the Hind III-M fragment. Western blot analysis of the fusion protein expressed in lysogenic *E. coli* (Y1089 strain) transfected with VEF clone had a molecular weight of 153 kD (Fig. 2). This suggested a fusion protein gene consisting of 39 Kd of the VEF carboxy terminal end and the 114 Kd beta-gal gene (Fig. 1c, 2). Since the VEF has a size of 104 Kd, the position of the VEF gene on a fine map of the Hind III-M fragment was predicted and a 3.5 kbp DNA portion was sequenced (Fig. 1b).

Sequence analysis showed an open reading frame of 2,703 bp DNA corresponding to the size of the VEF polypeptide at the predicted location of the VEF gene (Fig. 3). The deduced size of the polypeptide was 104,300 daltons and consisted of 901 amino acid residues. There are no sites for lipophilic modification (Lys/X/X/Cys/X/X/Asn). To determine the presence of the VEF gene among several isolates of baculovirus a 1.5 kbp portion of the VEF gene was probed onto a Southern blot of different virus DNA fragments digested with Hind III restriction enzyme under high stringency condition (12). The result showed that two granulosis virus DNAs, isolated from PuGV-H and HaGV, contained a sequence homologous to the TnGV VEF gene probe (Fig. 5a). DNA isolated from EeGV did not contain sequences homologous to the VEF gene probe. The restriction enzyme digestion pattern of DNA from TnGV, GVH, and HaGV were very similar, whereas EeGV exhibited a very distinct DNA Profile. The probe did not hybridize with DNAs from two nuclear polyhedrosis viruses (Fig. 5a). Temporal gene expression of the VEF gene was examined by Northern blot analysis of total RNA from TnGV-infected *T. ni* larvae at 3 and 6 days p.i. A probe with a size of 1.5 Kb KpnI-V fragment, a part of the VEF gene, showed no hybridization with RNAs at 3 days p.i. but showed strong hybridization with two RNA species with sizes of 2.7 Kb and 3.3 Kb at 6 days p.i. (Fig. 4). The TnGV-VEF present in the granulin fraction of alkaline dissolved OBs was resolved as a 104 Kd protein on a SDS-polyacrylamide gel. To determine the presence of high molecular weight polypeptides in granulin or polyhedrin fractions from six baculoviruses, these virus samples were analyzed by SDS-PAGE (Fig. 5b). In the granulin fractions from TnGV, GVH and HaGV, polypeptides with a size of 104 Kd, 106 Kd, and a complex of 110 Kd and 94 Kd were detected, respectively. The single high molecular weight polypeptide (106 Kd) from GVH appears to migrate on SDS-PAGE similar to the 104 Kd protein from TnGV (Fig. 5b and Y. Tanada, personal communication). The assignment of a VEF function to either the 94 or 110 kDa polypeptides from HaGV is not clear at this time. No polypeptides with a size of approximately 100 kDa were present in EeGV, TnSNPV, and AcMNPV. Three of the GV's examined, TnGV, GVH, and HaGV all infect the noctuid species *T. ni*, whereas EeGV grows only in the sphyngid species, *E. ello*.

KpnI and SalI subclones of the EcoRI-I fragment of PuGV-H, which contains the entire SF gene, were cloned into the KpnI and SalI sites of pUC 19 (40). This was followed by nested deletions from both ends of the subcloned DNA using the Exo/mung deletion kit (Stratagene). The nucleotide sequence was determined using the dideoxy chain termination method of Sanger et al. (32) as modified for use with the Sequenase sequencing kit (U.S. Biochemicals). Sequencing data were compiled and analyzed using the PCGENE (Intelligenetics) software package.

Neonate bioassays employing 3-5 hr old larvae were conducted according to the methods of Hughes et al. (28) except that the neonates were not preselected for vigor and the droplets were applied by means of a syringe equipped with a blunt needle (26). The inoculum contained 1×10^5 OB/ml of AcMNPV with 1.0 or 0.5 mg/ml of either TnGV VEF or PuGV-H SF and all larvae were assumed to have imbibed 10 nl of inoculum (27). After ingestion of the inoculum, larvae were transferred with a fine paintbrush into individual 35 ml cups containing high wheat germ diet. Controls consisted of neonates that imbibed either virus without any enhancing factor or water with food coloring. The test was conducted 2 times with 30 larvae/treatment in each test group.

In Vitro peritrophic membrane assay

PM were dissected from last instar *T. ni.* and *P. unipuncta* larvae, rinsed in deionized water to remove diet residue, and stored at -80°C . Thawed PMs were resuspended in 50 ml of digestion buffer (0.1 M Na_2CO_3 , pH 10.5) containing either 5 μg or 10 μg of SF or VEF. After incubation at 28°C for 1 hr, the PMs were washed in water, placed in 1X SDS-PAGE sample buffer and boiled for 5 min. Controls consisted of PMs treated in the same manner but without any enhancing factor. The protein composition of the treated and control PMs were analyzed by discontinuous SDS-PAGE (79) on a Mini-PROTEAN II (BioRad) at 200 volts for 35 min with a 10% separating gel. Gels were stained using the BioRad Silver Staining Kit according to the manufacturer's instructions.

Western blots.

Viral occlusion bodies were first dissolved in dilute alkali solution (0.1 M Na_2CO_3 , 0.01 M EDTA, 0.17 M NaCl, pH 10.9) for 15 minutes at room temperature. An equal volume of 2X SDS-PAGE sample buffer was added and the mixture heated in a boiling water bath for 7 minutes. Samples were separated by SDS-PAGE as described above. The proteins were then electrophoretically transferred to nitrocellulose paper following the methods of Towbin et al. (38). Western blots were analyzed using an anti-VEF/TrpE polyclonal antibody at a dilution of 1:5000 (23). Bands were visualized using an alkaline phosphatase conjugated secondary antibody (19).

SUMMARY OF RESULTS

For cloning and sequence analysis of VEF, two positive clones were identified from the approximately 6000 plaques screened with a α -VEF polyclonal antiserum. Both clones had identical inserts of 2.8 Kb mapped to the Hind III-M fragment of the TnGV genome (92.2 to 95.8 map units; Fig. 1a). Other TnGV fragments hybridizing to the clones included the 6.7 Kb EcoRI-K and the BamHI-FG doublet. Detailed maps of both TnGV Hind III-M and the insert DNA were generated using several restriction enzymes (Fig. 1b,c).

Western blot analysis using both an anti-VEF polyclonal antisera and an anti- β -galactosidase monoclonal antibody (Promega, Madison, WI) demonstrated that the fusion protein generated by lambda-F had a molecular weight of 153 Kd which presumably consisted of 39 Kd of VEF carboxy-terminal and 114 Kd of β -galactosidase protein (Fig. 2). The VEF gene was tentatively positioned on the Hind III-M fragment using this information.

Sequence analysis of approximately 3.5 Kbp of Hind III-M DNA revealed an open reading frame of 2703 bp (901 amino acids) encoding a protein with a predicted molecular weight of 104.3 Kda (Fig. 3). The predicted protein contains 12 candidate sites for N-linked glycosylation (ASN/X/SER or Thr) and no sites predicted for lipophilic modification (LYS/X/X/CYS/X/X/ASN). A consensus baculovirus late promoter (AT-AAG) occurred at -4 nt and a probable polyadenylation signal (AATAA) was found 2 nt downstream of the VEF ORF. The upstream region of the VEF gene contained three perfect repeats of the sequence TTACAAGA between -192 and -149 nt of the translation start site. Curiously, these repeats were similar to the baculovirus consensus sequence for hyperexpression described by Rohrman (5). However, in all three sequences, mismatches occurred at the invariable "T" of the "ATAAG" core late promoter motif. Changes at this position have been shown to eliminate transcriptional initiation (5). A comparison of the deduced

amino acid sequence of the VEF with both the NBRF and Swiss-Prot protein data bases did not reveal any similarity to known proteins.

The occurrence of the late core promoter sequence at -4 bp indicated that VEF should be expressed late in infections. This was demonstrated by isolating RNA from infected larvae at several times (3 days and 6 days) PI. Using a restriction fragment from within the VEF open reading frame (ORF) as a probe, strong hybridization was shown to 2 RNA species (2.7 and 3.3 Kb) at 6 days PI but none at 3 days PI (Fig. 4). The transcript size of 2.7 Kb agreed with the predicted transcription start and stop signals adjacent to the open reading frame.

For SF sequence analysis, approximately 3300 bp within the EcoRI-I fragment of PuGV-H was sequenced, in both directions, revealing a 2703 bp open reading frame (ORF; Fig. 6) with a calculated molecular weight of the protein of 104Kd. A consensus baculovirus late promoter motif (ATAAG; Ooi et al.) (30) was located at -8 to -4 nts relative to the ORF. A comparison of both the nucleotide and amino acid sequence with that of the VEF gene from TnGV revealed a 99.1% (Fig. 6) and 98.2% (Fig. 7) homology respectively. The only significant difference in homology between PuGV-H SF and TnGV VEF genes occurs between nucleotide +1962 and +1985. Two reciprocal frameshifts in the PuGV-H sequence have caused a 7 amino acid gap which shares no homology to the TnGV VEF protein sequence. Homology of the PuGV-H gene with the TnGV VEF gene was greater than 95% for 300 bp upstream of the gene. After this point, the homology decreases to 17.7%. From data analyzed thus far, the PuGV-H and TnGV sequence homology is greater than 99% for 155 nts downstream of the genes. A consensus baculovirus late promoter motif is located 35 nt upstream of the stop codon of the VEF and SF gene sequences, and 78 nt upstream of a potential ORF. This possible second ORF is located 43 nt downstream of the SF and VEF ORFs.

The enhancing protein from PuGV-H was purified from capsules in the same manner as the TnGV VEF. Approximately 330 mg of purified protein was isolated from 1.0×10^{12} OBs. Based on SDS-PAGE analysis, purified SF had a calculated molecular weight of 106Kd (Fig. 8). While this was in good agreement with the predicted molecular weight of 104Kd from other protein sequence analysis there is a repeatable difference in the migration pattern when compared to the TnGV VEF protein (Fig. 8).

The homology between the VEF and SF proteins suggested that the ability to enhance baculovirus infections should also be similar. This was tested by a neonate larval bioassay (Table 1) and an *in vitro* PM assay (Fig. 9). Enhancement of AcMNPV infections of *T. ni* larvae occurred with the VEF and SF proteins. The 2.4 fold enhancement of

TABLE 1

Effect of PuGV-H and TnGV Enhancing Factors on AcMNPV Infections of <i>Tricloplusia ni</i> Neonate Larvae*		
Enhancing Factor		Percent Mortality†
Source	ng/larva	
PuGV-H	10	95
PuGV-H	5	95
TnGV	10	95
	0	40

* All larvae were infected with 1 OB.

† This represents the average of two bioassays with 30 larvae per treatment. Non-virus control had no mortality.

infections by SF was identical to that seen in the VEF assays. In the *in vitro* PM assay, SF and VEF digested the same proteins in both the *T. ni* and *P. unipuncta* PMs. For the *T. ni* PM, 3 proteins of molecular weight 36.6Kd, 111.5Kd, and 98.2Kd present in the control lanes are absent in the SF and VEF treatment lanes (Fig. 9a). Protein bands found only in the treatment lanes include a predominant group of bands occurring between 71.6K and 58.8K and 2 lower molecular weight proteins of 31.2K and 23.3K (Fig. 9a). In *P. unipuncta*, 7 proteins are absent in the treatment lanes as compared to the control (Fig. 9b, lane 1). The molecular weight of the digested bands are 210.5Kd, 184.3Kd, 171.1Kd, 125.7Kd, 111.5Kd, 36.4Kd, and 32.0Kd. While there are 4 new protein bands of molecular weight 182.4Kd, 121.3Kd, 32.4Kd, and 24.6Kd common to both SF and VEF treatments, 4 unique proteins are also evident: 85.5K in VEF and 91.8K, 82.7K, and 80.0K in the SF treatment (Fig. 9b, lanes 2 and 3).

In order to ascertain the prevalence of the VEF gene within the Baculoviruses, 17 different baculoviruses (12 GVs and 5 NPVs) have been screened for VEF homologs using a polyclonal antiserum specific for the TnGV VEF protein (Fig. 10 and 11). Cross-reactive proteins were found in 7 GVs: *Cydia pomonella* GV (CpGV), *Estigmene acrea* GV (EaGV), HaGV, PrGV, PuGV, and StGV. This represents GVs which were isolated from 4 different families of Lepidoptera: Arctiidae, Noctuidae, Pieridae, and Tortricidae. EeGV, PuGV Oregon strain, *Plodia interpunctella* GV, *Pieris brassicae* GV, and *Spodoptera frugiperda* GV did not have any cross-reactive proteins. None of the NPVs (AcMNPV, *Anticarsia gemmatilis* MNPV, *Choristoneura fumiferana* NPV, *Helicoverpa zea* SNPV, and TnSNPV) reacted with the antiserum.

The identified VEF cross-reactive proteins could be subdivided based on the molecular weight of the proteins. PrGV, PuGV-H, StGV, and TnGV had the most common protein size of approximately 104Kd. HaGV had a slightly higher molecular weight (110Kd) while CpGV and EaGV had a significantly lower molecular weight of approximately 80Kd.

The cloning and sequencing of the SF gene from PuGV-H represents the second baculovirus enhancing factor to be sequenced to date. The high degree of homology between the PuGV-H and TnGV genes is unusual and indicates that there may be a strong selective pressure on the gene. Another possible explanation is that PuGV-H may be a variant of TnGV; however, this seems unlikely since the degree of homology decreases to 17.6% 300 bps upstream of the gene. In addition, there are significant differences in the restriction enzyme patterns of the two viral genomes (23). The identification of a second open reading frame may explain the high degree of homology (> 99%) observed downstream of the two genes. The effect of this downstream gene on the expression of VEF or SF is unknown. On SDS-PAGE, the SF and VEF proteins show a consistent difference in mobility. Since these proteins have near identical molecular weights it is possible that the two proteins may be processed or modified differently in the two hosts.

The results from both the neonate and *in vitro* PM assays demonstrate that the two proteins are very similar in activity. The observed differences in the digestion patterns of the PM proteins from both *T. ni* and *P. unipuncta* are probably due to quantitative differences in the amounts of the two enzymes. Evidence for this comes from the *T. ni* digests and the periodicity of the protein bands between 71.6Kd and 58.8Kd. The same bands are present in both the SF and VEF digests; however, the intensity of the bands differ. In the VEF digest (Fig. 9a, lane 2) the higher molecular weight bands predominate while in the SF digest (Fig. 9a, lane 3) the opposite is true. The data suggests a possible endoproteolytic type of cleavage in which the digestion in the SF reaction has proceeded further than in the VEF reaction.

Five baculoviruses were originally tested for the presence of VEF-homologous proteins by both DNA hybridization and SDS-PAGE analysis of dissolved occlusion bodies (Fig. 4A and B) Hind III genomic digest of the 5 baculovirus DNAs under low stringency conditions, using a restriction fragment with the VEF ORF as a probe, showed homology between TnGV and 2 other granulosis viruses (PuGV-H and HaGV). No apparent homology was seen to either EeGV, TnSNPV, or AcMNPV (Fig. 5A).

To date, a total of 8 GVs have been reported to have enhancing proteins. Seven of the proteins cross-react with a polyclonal antiserum specific for the VEF from TnGV. The other enhancing factor, which is found in *Xestia c-nigrum* (23), has not been tested with the antiserum. Enhancing proteins have now been identified in baculoviruses isolated from four families of Lepidoptera: Arctiidae, Noctuidae, Pieridae, and Tortricidae. Previously, enhancing factors had only been identified in GVs infecting Noctuidae. This data lends credibility to the hypothesis that these enhancing proteins are common in GVs and are important baculovirus proteins which assist in the initial stages (PM penetration and virion adsorption) of larval infections. Applicants' inability to identify cross-reacting proteins in the NPVs suggests that while these viruses may have proteins which are functionally related to the GV enhancing factors (1), they are unrelated in primary amino acid sequence.

The identified baculovirus enhancing proteins can be tentatively separated into 3 distinct groups based on molecular weight: 104Kd, 110Kd, and 80Kd. All of the research has concentrated on 2 enhancing factors from the same group, PuGV-H and TnGV (104Kd).

The absence of a protein in PuGV-O which does not cross-react to the VEF antiserum, confirms earlier reports indicating that PuGV-O does not contain an enhancing factor (6) and that differences exist in the capsular components of PuGV-H and PuGV-O (34,41).

It is important to note that the baculoviruses are just one of many insect pathogenic organisms that have evolved mechanisms, both behavioral and structural, to circumvent the PM (25) and *Babesia microti*, an intraerythrocytic piroplasm of the tick *Ixodes dammini*, has developed a complex "arrowhead" structure which secretes a series of digestive enzymes to enable passage through the PM (31).

The VEF and SF genes of the present invention can be used in engineering new viral pesticides with enhanced efficacy. For example, it can be used alone as biopesticide or in combination with known biological insecticides such as BT or with synthetic chemical insecticides. The gene product of this

invention can also be used to produce VEF or SF in any microbial production system, e.g. *E. coli*, *Bacillus* or *Streptococcus*. It can be introduced into a variety of hosts such as plants for protection against insects or microbes as biologically active agents.

The genes of this invention can be engineered to be expressed in transgenic plants and as insects feed on these plants, they would ingest a constant dose of the factor. While the exact effect of this on the insect is undetermined, it can be hypothesized that prolonged disruption of the peritrophic membrane (PM) may allow opportunistic microbes to infect and kill the insects. It was recently found that the viral factor increases the efficiency of Bt delta endotoxin by removing a major mechanical barrier--the PM.

The genes of the present invention have been found to play a significant role as a determinant of virulence at the initial stage of infection in insect hosts. Knowledge gained in cloning and sequencing the viral gene should prove useful in helping to unravel the mechanism(s) of enhanced virus infection by enhancement factors present within the occlusion body matrix.

Notwithstanding that reference has been made to particular preferred embodiments, it will be understood that the present invention is not to be construed as limited as such, but rather to the lawful scope of the appended claims. In other words, the subject invention includes not only the specific nucleotide sequences depicted herein, but also all equivalent nucleotide sequences coding for molecules with substantially the same biological activity of enhancing the infectivity of baculoviruses. The term "equivalent" is being used in ordinary patent usage here as denoting a nucleotide sequence which performs substantially as the nucleotide sequence identified herein to produce molecules with substantially the same biological activity in essentially the same kind of hosts. Within this definition are subfragments which have biological activity of enhancing the infectivity of baculoviruses.

Inasmuch as the protein, i.e., the gene product, of the present invention has been defined by means of deductive amino acid sequencing, c.f. Fig. 3, it is to be understood that for this particular protein, embraced herein, natural allelic variations exist and occur from individual to individual. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. All such allelic variations are included within the scope of the present invention.

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INFORMATION FOR SEQ ID NO:1

5 (i) (A) LENGTH: 3556 basepairs
 (B) TYPE: Nucleic with corresponding amino acid
 sequence (435-3140)
 (C) STRANDEDNESS: Doublestranded
 (D) TOPOLOGY: Circular

10 (ii) MOLECULE TYPE: Genomic DNA
 (iii) HYPOTHETICAL: No
 (iv) ANTISENSE: No

15 (v) FRAGMENT TYPE: N/A
 (vi) (A) ORIGINAL SOURCE: *Trichoplusia ni* granulosis virus
 (B)-(F) Not applicable

20 (vii) (A) LIBRARY: Lambda GT11
 (B) CLONE: HindIII-M
 (viii) (A) CHROMOSOME/SEGMENT: N/A
 (B) MAP POSITION: 92.2 - 94.2
 (C) UNITS: genome percent

25 (ix) FEATURE

1. (A) Mature protein
 (B) 435 bp to 3140 bp
 (C) Experimentally
 (D) Degrades special Peritrophic Membrane proteins
 Binds to midgut brush border

30 2. (A) Baculovirus very late promoter
 (B) 427 to 432 bp
 (C) N/A
 (D) N/A

35 3. (A) Possible glycosylation sites
 (B)

40	Site 1	65	to	67	amino acids
	Site 2	265	to	267	amino acids
	Site 3	305	to	308	amino acids
	Site 4	339	to	341	amino acids
	Site 5	349	to	351	amino acids
	Site 6	540	to	542	amino acids
45	Site 7	594	to	596	amino acids
	Site 8	595	to	597	amino acids
	Site 9	621	to	623	amino acids
	Site 10	642	to	644	amino acids
	Site 11	683	to	685	amino acids
50	Site 12	698	to	700	amino acids

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(C) Experimental
(D) Not known

SEQUENCE ID NUMBER: 1
SEQUENCE TYPE: Nucleotide with corresponding protein
SEQUENCE LENGTH: 3556 base pairs

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				145					150					155					
5	AAA	AAC	GCT	TTA	TTG	GAC	GTG	AAC	ATT	TTC	GAG	CTT	CAT	CAA	TTT	TAT		950	
	Lys	Asn	Ala	Leu	Leu	Asp	Val	Asn	Ile	Phe	Glu	Leu	His	Gln	Phe	Tyr			
				160					165					170					
	AAC	GAA	ATC	ATT	AAT	TAC	TAT	GAT	GAC	CTG	TGC	GGC	TTG	GTC	GAG	GAT		998	
	Asn	Glu	Ile	Ile	Asn	Tyr	Tyr	Asp	Asp	Leu	Cys	Gly	Leu	Val	Glu	Asp			
			175					180					185						
10	CCA	TAC	GCA	GAC	ACT	GTC	GAT	TCG	AAT	TTA	CCC	AAC	AAG	GCT	GCT	TTC		1046	
	Pro	Tyr	Ala	Asp	Thr	Val	Asp	Ser	Asn	Leu	Pro	Asn	Lys	Ala	Ala	Phe			
			190				195					200							
15	GTG	AAA	GCT	GAT	GCT	GGC	GGT	CCG	GGT	GGT	GCG	TAT	TAT	GGA	CCA	TTT		1094	
	Val	Lys	Ala	Asp	Ala	Gly	Gly	Pro	Gly	Gly	Ala	Tyr	Tyr	Gly	Pro	Phe			
	205					210					215					220			
	TGG	ACG	GCA	CCG	GCG	AGC	TCA	AAC	CTT	GGT	GAT	TAC	CTC	AGA	ATA	TCG		1142	
	Trp	Thr	Ala	Pro	Ala	Ser	Ser	Asn	Leu	Gly	Asp	Tyr	Leu	Arg	Ile	Ser			
				225						230					235				
20	CCG	ACC	AAC	TGG	ATG	GTA	ATT	CAC	GAG	CTG	GGT	CAT	GCA	TAC	GAT	TTT		1190	
	Pro	Thr	Asn	Trp	MET	Val	Ile	His	Glu	Leu	Gly	His	Ala	Tyr	Asp	Phe			
				240					245				250						
25	GTG	TTT	ACC	GTC	AAC	ACT	ATA	CTC	ATT	GAA	ATT	TGG	AAC	AAC	TCT	TTA		1238	
	Val	Phe	Thr	Val	Asn	Thr	Ile	Leu	Ile	Glu	Ile	Trp	Asn	Asn	Ser	Leu			
			255					260					265						
	TGC	GAT	CGC	ATC	CAA	TAC	AAG	TGG	ATG	AAC	AAA	ATT	AAA	AGA	CAA	CAA		1286	
	Cys	Asp	Arg	Ile	Gln	Tyr	Lys	Trp	MET	Asn	Lys	Ile	Lys	Arg	Gln	Gln			
		270					275					280							
	CTG	GCT	CGC	GTC	TAT	GAA	AAT	AGA	CGA	CCG	CAG	AAA	GAG	GCG	ACC	ATT		1334	
	Leu	Ala	Arg	Val	Tyr	Glu	Asn	Arg	Arg	Pro	Gln	Lys	Glu	Ala	Thr	Ile			
	285					290					295					300			
35	CAG	GCG	CTG	ATC	GAC	AAT	AAC	AGC	CCG	TTC	GAT	AAT	TGG	GGC	TTT	TTT		1382	
	Gln	Ala	Leu	Ile	Asp	Asn	Asn	Ser	Pro	Phe	Asp	Asn	Trp	Gly	Phe	Phe			
				305						310					315				
	GAG	AGG	CTG	ATA	ATA	TTC	ACG	TGG	CTG	TAC	AAC	CCG	CAA	AGA	GGA	CTA		1430	
	Glu	Arg	Leu	Ile	Ile	Phe	Thr	Trp	Leu	Tyr	Asn	Pro	Gln	Arg	Gly	Leu			
				320					325				330						
	GAC	ACA	TTG	CGT	AAC	ATC	AAC	CAT	TCG	TAC	AGG	GTG	CAC	GCC	ACC	CGC		1478	
	Asp	Thr	Leu	Arg	Asn	Ile	Asn	His	Ser	Tyr	Arg	Val	His	Ala	Thr	Arg			
			335					340					345						
45	AAC	TCT	TCT	ATA	CCG	TAC	CCG	CAA	ATA	TGG	TCA	TGG	CTA	ACG	ACT	TCT		1526	
	Asn	Ser	Ser	Ile	Pro	Tyr	Pro	Gln	Ile	Trp	Ser	Trp	Leu	Thr	Thr	Ser			
			350				355					360							
50	GCT	TAC	GAC	AAC	TTT	TGG	TTA	TAT	TTT	AAT	TTG	GTA	GGC	GTG	TAC	CCG		1574	

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	Ala	Tyr	Asp	Asn	Phe	Trp	Leu	Tyr	Phe	Asn	Leu	Val	Gly	Val	Tyr	Pro	
	365					370					375					380	
5	GCA	GAC	TTT	TAC	GTA	AAC	GAA	CAC	AAC	AAA	GTT	GTT	CAT	TTC	AAT	CTA	1622
	Ala	Asp	Phe	Tyr	Val	Asn	Glu	His	Asn	Lys	Val	Val	His	Phe	Asn	Leu	
					385					390					395		
	CAC	TTG	AGA	GCT	TTG	GCG	TTG	GGG	CAG	AGT	GTG	CGT	TAT	CCC	ATT	AAA	1670
10	His	Leu	Arg	Ala	Leu	Ala	Leu	Gly	Gln	Ser	Val	Arg	Tyr	Pro	Ile	Lys	
				400				405					410				
	TAT	ATA	ATT	ACA	GAC	TTT	GAT	CTG	GTG	AGC	AAA	AAC	TAC	GAC	ATT	AAA	1718
	Tyr	Ile	Ile	Thr	Asp	Phe	Asp	Leu	Val	Ser	Lys	Asn	Tyr	Asp	Ile	Lys	
				415				420					425				
15	CAG	TAT	TTA	GAG	AGT	AAT	TTC	GAT	CTG	GTT	ATA	CCA	GAA	GAA	TTG	CGG	1766
	Gln	Tyr	Leu	Glu	Ser	Asn	Phe	Asp	Leu	Val	Ile	Pro	Glu	Glu	Leu	Arg	
				430			435					440					
	CAG	ACC	GAT	TTG	TTG	GCG	GAC	GTG	AGG	GTG	GTT	TGT	GTG	ATT	GAC	GAT	1814
20	Gln	Thr	Asp	Leu	Leu	Ala	Asp	Val	Arg	Val	Val	Cys	Val	Ile	Asp	Asp	
						450					455					560	
	CCG	TCG	CAG	ATT	GTG	GGC	GAA	CCG	TTT	AGC	GTG	TAC	GAC	GGG	AAC	GAG	1862
	Pro	Ser	Gln	Ile	Val	Gly	Glu	Pro	Phe	Ser	Val	Tyr	Asp	Gly	Asn	Glu	
					465					470					475		
25	CGA	GTG	TTC	GAG	AGT	ACG	GTG	GCC	ACG	GAC	GGA	AAC	ATG	TAT	CTG	GTG	1910
	Arg	Val	Phe	Glu	Ser	Thr	Val	Ala	Thr	Asp	Gly	Asn	MET	Tyr	Leu	Val	
				480				485						490			
30	GGC	GTG	GGT	CCG	GGA	GTG	TAC	ACG	TTG	CGT	GCG	CCA	CGC	GGC	AAA	AAC	1958
	Gly	Val	Gly	Pro	Gly	Val	Tyr	Thr	Leu	Arg	Ala	Pro	Arg	Gly	Lys	Asn	
				495				500					505				
	AAA	CGC	TAC	AAA	CTC	CAT	TTG	GCA	CAT	TCG	CCC	AGA	GAG	CCC	GTT	CAT	2006
35	Lys	Arg	Tyr	Lys	Leu	His	Leu	Ala	His	Ser	Pro	Arg	Glu	Pro	Val	His	
				510			515					520					
	CCG	GCC	AAC	GAC	CAC	ATG	TAT	CTG	CTC	GTG	ACG	TAT	CCC	TAC	TAC	AAT	2054
	Pro	Ala	Asn	Asp	His	MET	Tyr	Leu	Leu	Val	Thr	Tyr	Pro	Tyr	Tyr	Asn	
						530					535					540	
40	CAA	ACG	TTG	ACA	TAC	ACA	CCG	TAC	GTA	AAT	TCT	GAC	CTA	GCC	GTC	GAC	2102
	Gln	Thr	Leu	Thr	Tyr	Thr	Pro	Tyr	Val	Asn	Ser	Asp	Leu	Ala	Val	Asp	
					545				550						555		
	ATG	GCT	CAT	TTG	TTC	GGC	AGC	AAC	GAT	CGT	AGG	TAT	GTA	GCC	ACG	ATA	2150
45	MET	Ala	His	Leu	Phe	Gly	Ser	Asn	Asp	Arg	Arg	Tyr	Val	Ala	Thr	Ile	
				560					565					570			
	TAT	TTC	AAT	CCA	TTC	GAA	CAA	ACA	GTC	ACC	GTA	CAT	CTA	AAC	AAT	ATT	2198
	Tyr	Phe	Asn	Pro	Phe	Glu	Gln	Thr	Val	Thr	Val	His	Leu	Asn	Asn	Ile	
				575				580					585				
50																	
55																	

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	CGT	GCC	GGT	CGT	GAA	AAC	AAC	ACT	ACC	CTG	TAC	TTT	GAA	ATG	GTA	ATT	2246
	Arg	Ala	Gly	Arg	Glu	Asn	Asn	Thr	Thr	Leu	Tyr	Phe	Glu	MET	Val	Ile	
	590					595						600					
5	AGC	AAC	CCG	TTC	AAC	GGG	CAG	AGC	CAA	ACT	TTC	ACT	ATA	CTC	GAA	GAC	2294
	Ser	Asn	Pro	Phe	Asn	Gly	Gln	Ser	Gln	Thr	Phe	Thr	Ile	Leu	Glu	Asp	
	605				610						615					620	
	AAT	CCC	ACT	TTA	CGA	CAA	GGC	TAC	TAC	AAA	TTT	GAC	GTG	GTC	ACG	TAC	
10	Asn	Pro	Thr	Leu	Arg	Gln	Gly	Tyr	Tyr	Lys	Phe	Asp	Val	Val	Thr	Tyr	
					625					630					635		
	AGC	TCC	ATA	AGG	CTG	AAT	ATG	AGC	GTC	GCG	GGT	CGG	CTA	TTA	TTT	CGG	
	Ser	Ser	Ile	Arg	Leu	Asn	MET	Ser	Val	Ala	Gly	Arg	Leu	Leu	Phe	Arg	
				640					645					650			
15	CGA	TAC	ATT	TTT	GCC	GGA	GGT	ACC	ACC	ACG	CTG	ACC	ATG	TTC	CCA	AAT	2438
	Arg	Tyr	Ile	Phe	Ala	Gly	Gly	Thr	Thr	Thr	Leu	Thr	MET	Phe	Pro	Asn	
			655					660					665				
	CAA	GTA	CTT	GAG	CCC	AAT	TTG	TTT	CCA	GAC	GGT	TCC	GCC	TTG	AAT	AGG	
20	Gln	Val	Leu	Glu	Pro	Asn	Leu	Phe	Pro	Asp	Gly	Ser	Ala	Leu	Asn	Arg	2486
		670					675					680					
	ACA	TTG	GCA	CGA	CTA	AGA	GAA	CAG	GCC	GCC	TTC	CTA	GAT	AAT	TAT	TCA	
	Thr	Leu	Ala	Arg	Leu	Arg	Glu	Gln	Ala	Ala	Phe	Leu	Asp	Asn	Tyr	Ser	
	685					690					695					700	
25	CAA	CTT	ATG	TAT	ATT	GAA	AAC	GAG	TTG	CGC	GAC	ACG	ATT	TAT	TTG	GCC	2582
	Gln	Leu	MET	Tyr	Ile	Glu	Asn	Glu	Leu	Arg	Asp	Thr	Ile	Tyr	Leu	Ala	
					705					710					715		
	TCC	CAG	TTG	GTA	GAT	CCT	GCG	TCA	GAC	GAA	TTT	GTA	AAG	TAT	TAT	CCA	
30	Ser	Gln	Leu	Val	Asp	Pro	Ala	Ser	Asp	Glu	Phe	Val	Lys	Tyr	Tyr	Pro	2630
				720					725					730			
	GAC	TAC	TTC	AGA	GAT	CCG	CAC	ACG	TAC	GTG	TAC	TTG	TTT	CGT	TTC	AGA	
	Asp	Tyr	Phe	Arg	Asp	Pro	His	Thr	Tyr	Val	Tyr	Leu	Phe	Arg	Phe	Arg	
35			720					740					745				2678
	GGT	CTG	GGT	GAT	TTC	GTG	TTA	TTA	GAC	TTG	CAG	ATT	GTA	CCA	TTG	CTA	
	Gly	Leu	Gly	Asp	Phe	Val	Leu	Leu	Asp	Leu	Gln	Ile	Val	Pro	Leu	Leu	
		750					755					760					
40	AAT	TTG	GCC	ACT	GTA	CGT	ATA	GCC	AAC	ATC	CAA	AAC	GGT	CCC	CAC	TCG	2774
	Asn	Leu	Ala	Thr	Val	Arg	Ile	Ala	Asn	Ile	Gln	Asn	Gly	Pro	His	Ser	
	765					770					775					780	
	TAC	TTC	GAT	ACT	TTG	TAT	TTT	AAA	GTG	GAG	TTG	CGC	GAC	ACA	AAC	GGT	
	Tyr	Phe	Asp	Thr	Leu	Tyr	Phe	Lys	Val	Glu	Leu	Arg	Asp	Thr	Asn	Gly	
45					785					790					795		2822
	GCG	ATT	GTG	TTT	TCG	TAT	TCG	CGC	CGT	GGC	AAC	GAG	CCG	ATG	ACA	CCC	
	Ala	Ile	Val	Phe	Ser	Tyr	Ser	Arg	Arg	Gly	Asn	Glu	Pro	MET	Thr	Pro	
				800					805					810			
50																	
55																	

	GAA CAC CAT AAA TTT GAA GTG TAC AGT GGT TAC ACC GTA GAA TTG TTC	2918
	Glu His His Lys Phe Glu Val Tyr Ser Gly Tyr Thr Val Glu Leu Phe	
	815 820 825	
5	ATG CGG GAA CCC GGT AAT CGA TTA CAA TTG ATT GTG AAC AAA ATG CTT	2966
	MET Arg Glu Pro Gly Asn Arg Leu Gln Leu Ile Val Asn Lys MET Leu	
	830 835 840	
10	GAC ACA GCG TTG CCG TCT ACT CAA AAC ATT TTC GCT CGC ATC ACC GAC	3014
	Asp Thr Ala Leu Pro Ser Thr Gln Asn Ile Phe Ala Arg Ile Thr Asp	
	845 850 855 860	
15	ACT CAA TTA GTG GTG GGG GAT ACG AGC ATT GAA GAT AAC CTT GTA ACG	3062
	Thr Gln Leu Val Val Gly Asp Thr Ser Ile Glu Asp Asn Leu Val Thr	
	865 870 875	
	AGT ATT AAT GTA GAT TGT GGC GAC GAC GAC AAC CAA AAG ATA AGA GTT	3110
20	Ser Ile Asn Val Asp Cys Gly Asp Asp Asn Gln Lys Ile Arg Val	
	880 885 890	
	GTG GAA ACG TTA AAA ATG ATA GCG TTC TAA TAACGTTCAA CAGTCAGTTA	3160
	Val Glu Thr Leu Lys MET Ile Ala Phe	
	895 900	
25	TCGACTGTCG CCGCGACGAC ATGACACTGG TGGGTGTAGT AGTTTGCGTG CTGTTGTTAT	3220
	CGTCTGTAGA CGGTTATTCG TTTTATTCGT CGATTGAAGC CCTGCTTTTG AACGATCGCA	3280
	CACAACCTTG CATAGGCGAC TGTTACGAAC GCAATGGCCA GCATTTGTGT GCCAGCACGT	3340
	GGTCGGGATC AGAGTCTCGG TGCATAAGTG TTTTCAACAA GACCAAACAC TATCGTACGG	3400
	AGACTAACGG AAAATGCATA AGTAACTGTG CCAACTTCAA CAACTACGCC CACGAATGGT	3460
30	GTGCCGTGTC CCGGTCGAAA TGGGGCCGTT GCAGCAGACG ACTGGCGCTC ACAGCGACAC	3520
	GAACACACGC CACCCACAAC AAGTTCAAGA CATGTG	3556

35 Claims

1. A purified baculovirus protein designated as an enhancin and found in granulosi viruses within the viral occlusion body, said viruses being isolated from one of the following families of Lepidoptera Arctiidae, Noctuidae, Pieridae and Tortricidae, said protein being characterized by having a disruptive effect on the insect peritrophic membrane proteins by interacting with the midgut epithelium in such a manner as to permit the increased adsorption, penetration, and uptake of virus particles by midgut cells with a concomitant increase in host mortality.
2. An enhancin of claim 1 having a molecular weight between 80 Kd and 110 Kd.
3. The enhancin of claim 1 having molecular weight of 104 Kd.
4. An isolated and purified DNA from a granulosi virus comprising a DNA encoding the 901 amino acid residue polypeptide as shown in figure 3 and which polypeptide possesses the biological activity of enhancing baculovirus infectivity.
5. The DNA of claim 4 comprising the DNA of figure 3 from base +1 to base +2703 which is an open reading frame of 2703 base pairs encoding a protein with a molecular weight of 104 Kd.
6. The DNA of claim 4 comprising an allele encoding the 901 amino acid polypeptide.
7. An insecticide comprising a polypeptide characterized by having the amino acid and nucleotide sequence of figure 3.

8. A biopesticide comprising an enhancin of claim 1.
9. A composition comprising an enhancin of claim 1 and a pesticide.
- 5 10. A toxicant composition comprising an enhancin of claim 1 and a biological insecticide.
11. A toxicant composition comprising an enhancin of claim 1 and a synthetic chemical insecticide.
12. A microbial or cellular protein system for the production of an enhancin of claim 1.
- 10 13. The microbial system of claim 12 in which the microorganisms are selected from the group consisting of E. coli, Bacillus, Streptococcus, and a Baculovirus expression system.
14. Process of introducing an enhancin of claim 1 into plants for protection against insects.
- 15 15. Process of introducing an enhancin into microbes useful as biologically active agents.

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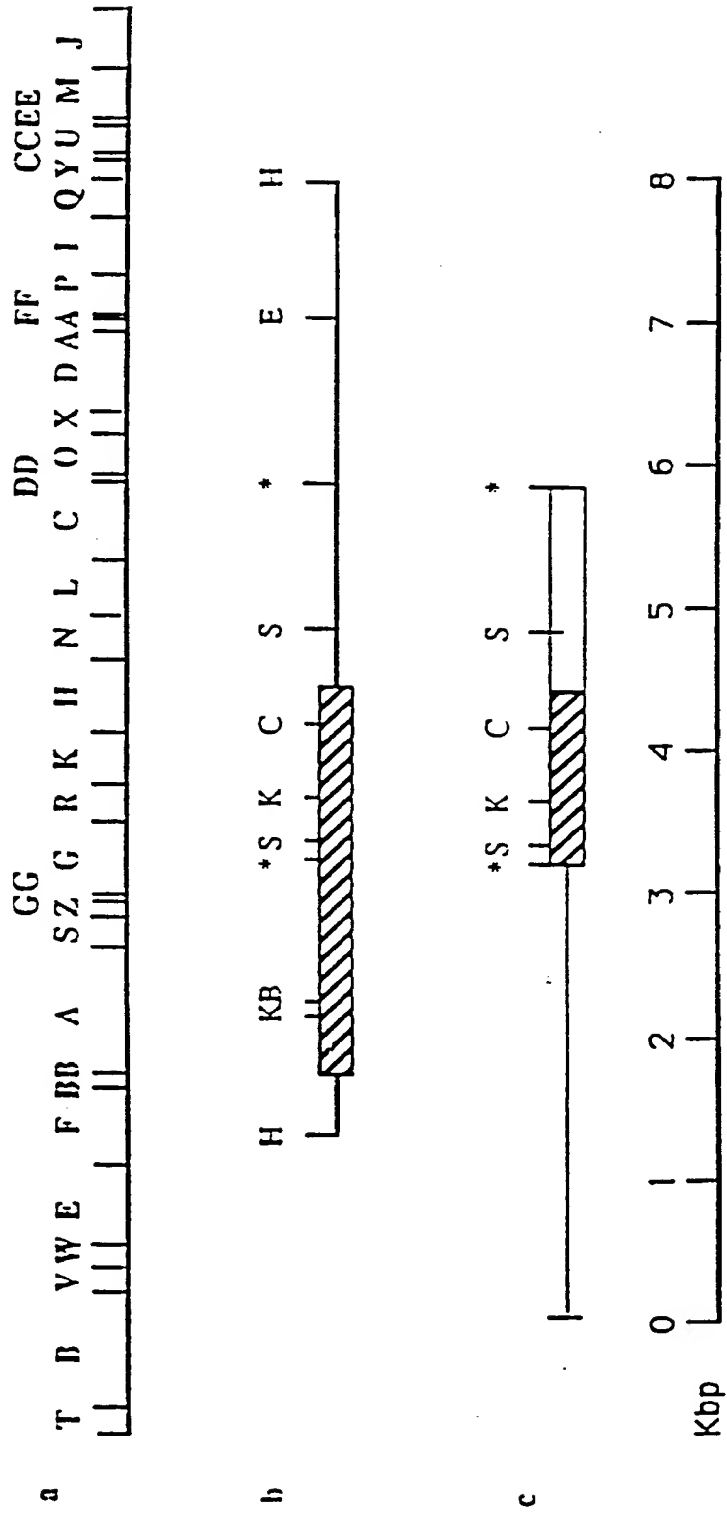


FIG. 1

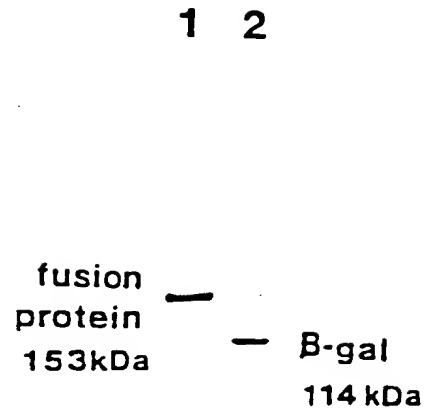


FIG. 2

[illegible]

FIG. 3

CGGAGCTGTACAGTTCGTCGGCCGCGGCAAAACAGGCTACAACTCCATTGGCACAATTCGCCAGACAGCCGTTTCATCCGCCCAACGACCAATGTATCTCTGCTGCTACGCT +1596
 P G V Y T L R A P R G K N K R Y K L H L A H S P R E P V H P A N D H M Y L L V T
 ATCCCTACTACAACTCAACGCTTGACATACACACCTGACGTAATTCGACCTAGCCGTCGACATGGCTCATTGTTCCGAGCAAGCATGCTAGGTATGTAGCCACCAATATATTCATC +1716
 Y P Y Y N Q L L T Y T P Y V N S D L A V D H A H L F G S N D R R Y V A T I Y F N
 CATTCGAACAAACAGTCACCGTACATCTAAACAAATATTCGTCGGCTCTGAAACAAACACTACCTGCTACTTTGAATGGTAAATTAGCAACCCGTTCAACGGCCAGACCCAACTTTCA +1836
 P F E Q T V T V H L N M I R A G R E N N T I L Y F E M V I S N P F N G Q S Q T F
 CTATCTCAAGACAAATCCCACTTTACGACAAAGGCTACTACAAATTTGACGTCGTCACGTCAGCTCCATAGGCTGAATATGACGCTCCGGCTCGGCTATTATTTCCGGATACATTT +1956
 T I L E D V P L R Q G Y Y K F D V V T Y S S I R L N M S V A G R L L L F R R Y I
 ITCCGCGAGGTACACACGCTGACCATGTTCCCAATCAAGTACTTGACCCCAATTTGTTCCAGAGGTTCCGCTTCGATAGACATTTGGCAGCACTAGACAAAGCCGCTTCC +2076
 F A C S T T L T M F P N Q V L E P N L F P D G S A L N R T L A R L R E Q A A F
 TACATAATTTACAACTTATGTATATTGAAACGAGTTCCGCGACACGATTTATTTGGCTCCCACTGGTAGATCTGCTCGCTCAGACGAATTTGTAAGTATATTCAGACTACTTCA +2196
 L D N Y S Q L H Y I E N E L R D T I Y L A S Q L V D P A S D E F V K Y Y P D Y F
 CAGATCCGACACGTCAGTCTGCTTTGACAGCTCTGGCTGATTTCTGTTATTACACTTCAGATTCAGATTTGCTAAATTTCCGCCACTGTACCTATAGCCCAACATCCAA +2316
 R D P H T Y V Y L F R F R G L G D F V L L D L Q I V P L L N L A T V R I A N I Q
 ACGTCCCACTGCTACTTCGATCTTTTAAAGTGGAGTTCCGCGACACAAAGGTCGCTGCTTTGTTGTTATTCGGCCGCTGGCAAGGAGCCGATGACACCCCAACACCATTA +2436
 N G P H S Y F D T L Y F K V E L R D T N G A I V F S Y S R R G N E P M T P E H H
 AATTTGAAGTGTACAGTGGTACAGGTAGAAATGTTTCATCCGCAACCCGTAATCCATTAACAAATGTTGCAACAAATGCTTCAACAGCGTTCCGCTGTACTCAAAACATTTTCG +2556
 K F E V Y S G Y T V E L F M R E P G N R L Q L I V N K M L D T A L P S T Q N I F
 CTCCCATCACCCACACTCAATTAAGTGGTGGGATACGAGCAATTAAGATTAACCTTGTAAAGATTTAAATGTAGATTTGTCGGCAGCAGCAACCAAAAGATTAAGATTTGTTGGAAAGCT +2676
 A R I T D T Q L V V G D T S I E D N L V T S I N V D C G D D N Q K I R V V E T
 TAAAAATGATAGGCTTCAATACAGTTCACAGTTCAGTATCCACTGTCCGCGCGACGACATGACACTGGTGGGTGTAGTATGTTTGGCTGCTGTTGTTATCGTCTGTACAGCGTTATTCG +2796
 L K H I A F - -
 TTTTATTCGCTGATGAGCCCTCTTTTCAGCGATTCGCACAACTTTTCATAGCCGACTGTTACGAACGCAATGGCCACGCAATTTGTGTCACGACGCTGCTCGGCATCAGAGTCTTCG +2916
 TGCATNAAGTGTTCACAAAGACCAACACTATCGTACGAGAGCTAAACGAAATGTCATAAGTAACTGTCCCACTTCANCAACTACGCCCAACGAATGCTGTGCTGCTGCTCCCGGTCGCA +3036
 TGGGCGCGTTCGACAGACGACTCGCGCTCACAGCGACACGGAACACACGCCCAACAAAGTTCAACAGCATGTG +3112

FIG. 3 (continued)

3 days p.l.
6 days p.l.

-946
-746
-440
-237
-135
-024

FIG. 4



FIG. 5a

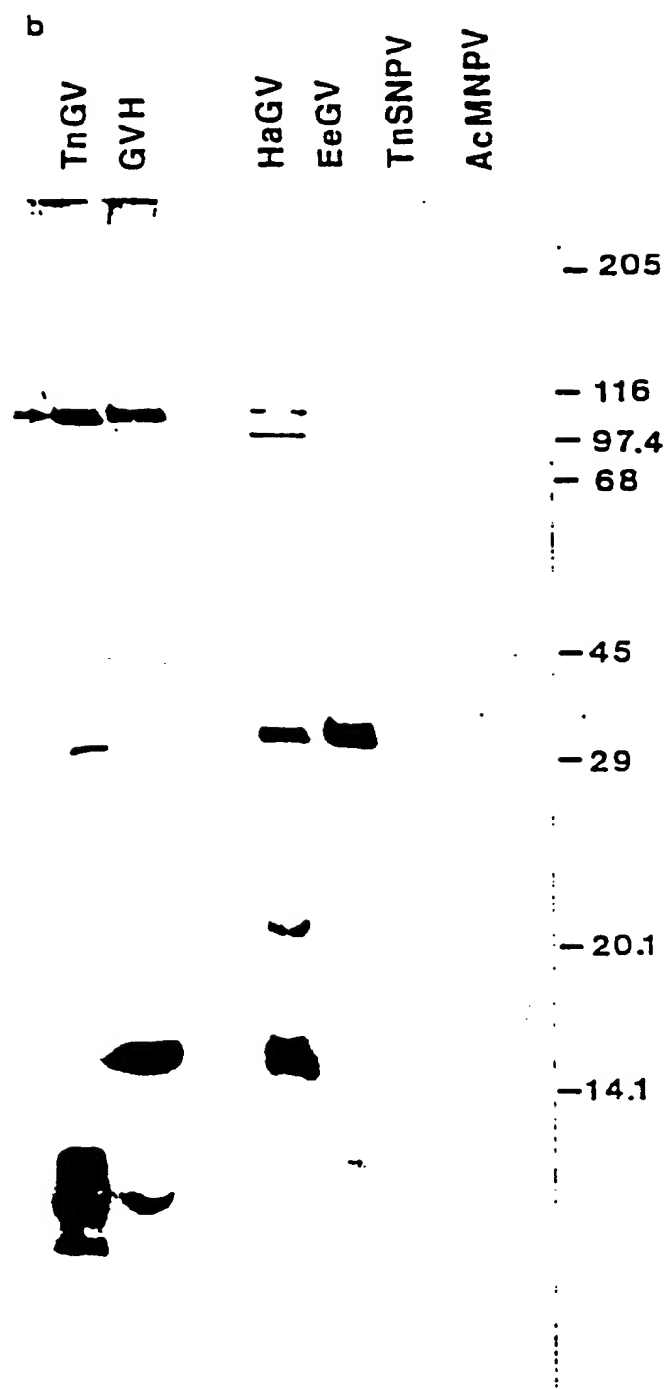


FIG 5b

[illegible]

FIG. 6

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Fig. 7

PuGV-H - MSYKVIVPATVVPFWLRVGZEWIFARHRRTEVGVVLPANTKFRVRADFSRAGFTRPVIVR -60
 TrGV - -----
 PuGV-H - LLNNNRNTEREINLNDQWMEVEHAHESVPFVDWPFVGERNIMAEVYFEIDGPHIPLFVYV -120
 TrGV - -----S-----L---K-T-----
 PuGV-H - FNTRPVEHFKSEYRQSSSGYCFLYLDLVCMVPPASKNALLDVNIFELHQFYNEIINYD -180
 TrGV - -----
 PuGV-H - DLCGLVEDPYADTVDSNLPNKAFVKADAGGPGGAYYGPFWTAPASSNLGDYLRIPTNW -240
 TrGV - -----
 PuGV-H - MVIHELGHAYDFVFTVNTILIEIWNNSLCDRIQYKWMNKTKRQQLARVYENRRPQKEATI -300
 TrGV - -----I-----
 PuGV-H - QALIDNNSPFDNWGFFERLIIFTWLYNPQRGLDTRLNINHSYRVHATRNSIPYQIWSW -360
 TrGV - -----
 PuGV-H - LTT SAYDNFWLYFNLVG VYPADFYVNEHNKVVFHNLHLRALALGQSVRYPIKYIITDFDL -420
 TrGV - -----
 PuGV-H - VSKNYDIKQYLESNFDLVIPEELRQTDLLADV RVVCVIDDPSQIVGEPFSVYDGNERVFE -480
 TrGV - -----
 PuGV-H - STVATDGNMYLVGVGPGVYTLRAPRGKXKRYKLHLAHSREFVHPANDHMYLLVTYPYYN -540
 TrGV - -----
 PuGV-H - QTLTYTPYVNSDLAVDMAHLFGSNDRRYVATIIYFNPFQTVTVHLNNIRAGRENNTLYF -600
 TrGV - -----
 PuGV-H - EMVLENPFNGQSQTFTILEDNPTLRQGYKFDVVTYSSIRLNMSVAGRLLFGDTFLPEGT -660
 TrGV - -----RRYIFAG--
 PuGV-H - TTLTMFPNQVLEPNLFPDGSALNRTLARLREQAAFLDNYSQLMYIENELRDSIYLASQLV -720
 TrGV - -----T-----
 PuGV-H - DPASDEFVKYYPDYFRDPHTYVYLFRFRGLGDFVLLDLQIVPLLNLATVRIANNHNGPHS -780
 TrGV - -----IQ-----
 PuGV-H - YFDTLYFKVELRDTNGAIVFSYSRRGNEPMTPEHHKFEVYSGYTVELFMREPGNRLQLIV -840
 TrGV - -----
 PuGV-H - NKMLDTALPSTQNI FARITDTQLVVGDTSIEDNLVTSINVDCGDDDNQKIRVETLKMIA -900
 TrGV - -----
 PuGV-H - FZ -902
 TrGV - --

Fig. 8

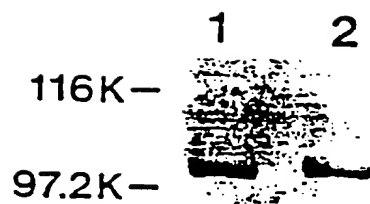


Fig 9

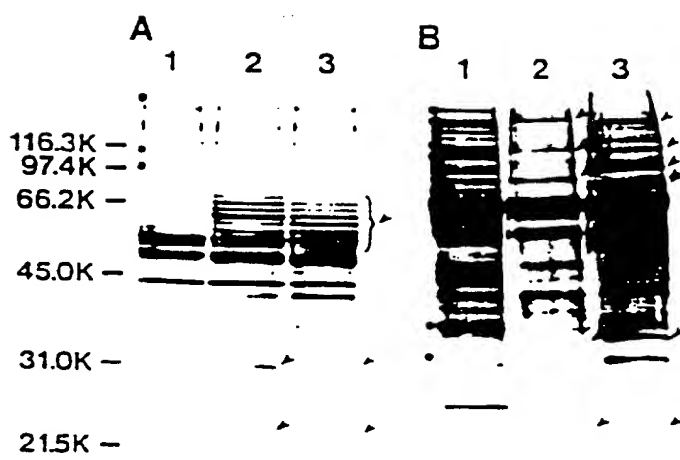


Fig. 10

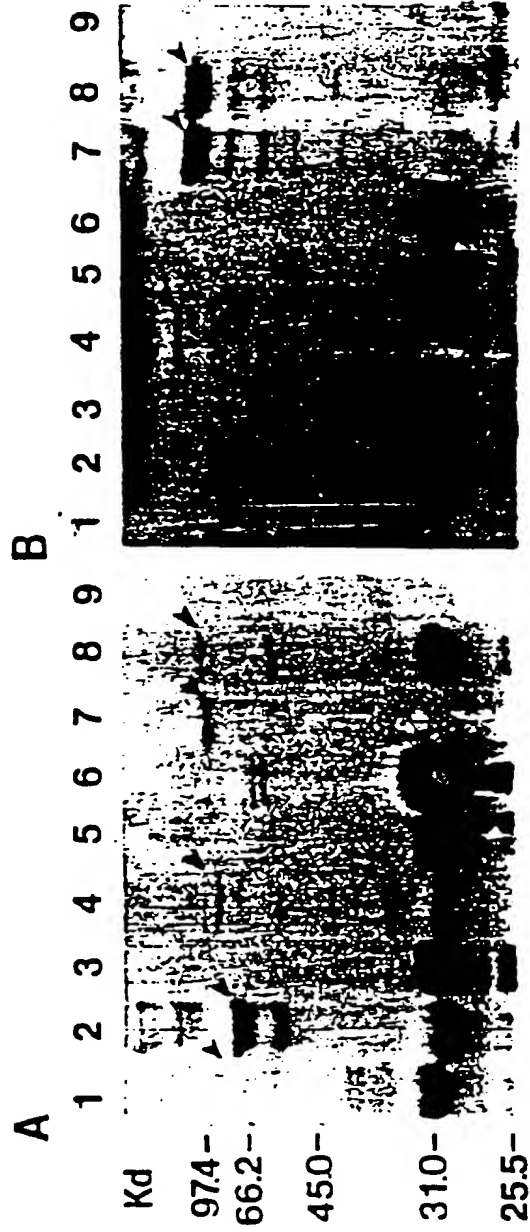
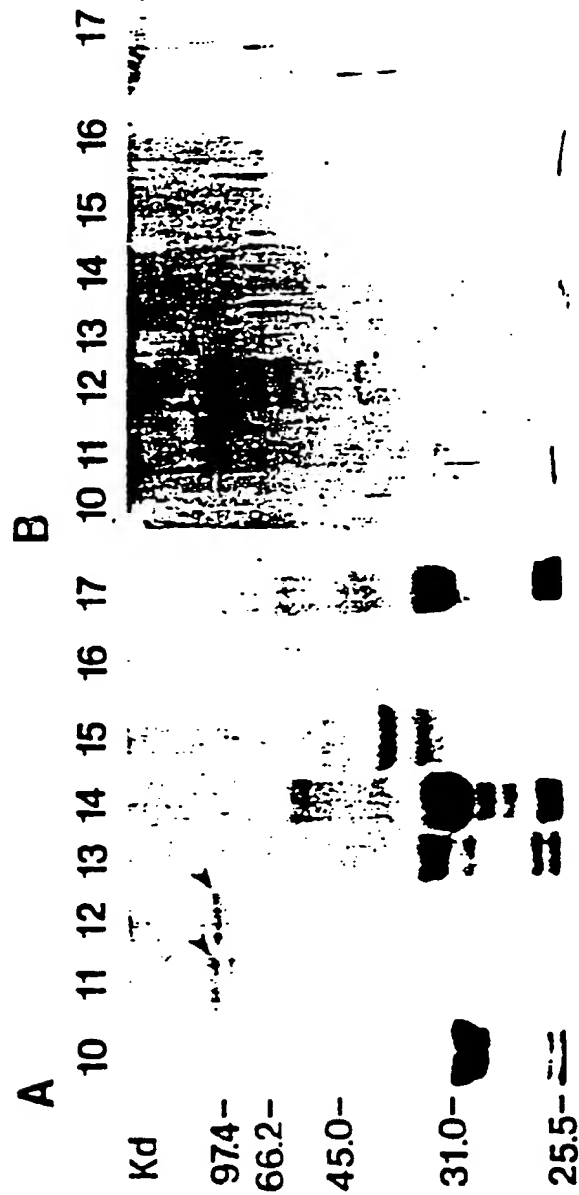


Fig. 12





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EUROPEAN SEARCH REPORT

Application Number

EP 93 11 7929

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	EP-A-0 502 236 (BOYCE THOMPSON INSTITUTE FOR PLANT RESEARCH) 9 September 1992 * Whole document; specifically Fig. 3 *	1-15	C07K15/00 C12N15/34 A01N63/00
X,D	J. GENERAL VIROLOGY vol. 72, no. 11, November 1991, pages 2645 - 2651 YOSHIFUMI HASHIMOTO ET AL. 'Location and nucleotide sequence of teh gene encoding the viral enhancing factor of the Trichoplusia ni granulosis virus' * "Methods" and sequence of Fig. 2 *	1-15	
X	EP-A-0 384 294 (BOYCE THOMPSON INST. FOR PLANT RESEARCH) 29 August 1990 * Claims 1 to 15 and Fig. 3 *	1-15	
X	EP-A-0 336 341 (BOYCE THOMPSON INST. FOR PLANT RESEARCH) 11 October 1989 * Page 3, line1-16 and claims 1-9 *	1-3,9-11	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
X,D	& US-A-4 973 667	1-3,9-11	C07K
A,D	J. INVERTEBRATE PATHOLOGY vol. 27, no. 1, January 1976, pages 115 - 124 S. HARA ET AL. 'Isolation and characterisation of synergistic enzyme ...' -----		
The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 01 FEBRUARY 1994	Examiner Germinario C.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document I : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document			

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Datum/Date

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Zeichen/Ref./Ref.

N.83606 PJC

Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°

00908824.6-2406/AU0000181

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION

COMMUNICATION

The European Patent Office herewith transmits the supplementary partial European search report under Rule 46(1) EPC relating to the above-mentioned European patent application.

Copies of the documents cited in the search report are enclosed.

The applicant's attention is drawn to the following:

The search Division informs the applicant that if the European search report is also to cover inventions other than the invention first mentioned in the claims, a further search fee must be paid for each of these inventions, within ONE MONTH after notification of this communication.

If the application has been filed up to 30 June 1999, the search fee in force before 01 July 1999 (EUR 869,-) or the equivalent applicable on the date of payment is payable.

This applies also to the search fees requested under Rule 46(1) EPC.

See also OJ EPO 06/1999, 405.

☐ The abstract was modified by the Search Division and the definitive text is attached to the present communication.

☒ Additional set(s) of copies of the documents cited in the European search report is (are) enclosed as well.



Note to users of the automatic debiting procedure:

Unless the EPO receives prior instructions to the contrary, the search fee(s) will be debited on the last day of the period for payment. For further details see the Arrangements for the automatic debiting procedure, Supplement to OJ EPO 02/1999.

REGISTERED LETTER



The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1,2,7-10,15-21 partially; 3-6,11-14 completely

Transformed plant comprising at least one polynucleotide molecule comprising a nucleotide sequence encoding a constituent protein from a spindle body or spindle-like body from an insect virus, said protein being fusolin or fusolin-like protein, and subject-matter related thereto.

Method for controlling or preventing damage caused to plants from feeding insects, comprising applying to said plant a feed bait composition comprising fusolin or fusolin-like proteins, with the proviso that the feed bait composition does not further comprise a nuclear polyhedrosis virus, wherein said composition is applied before, after or together with an insecticidal and/or biological agent.

Method for controlling or preventing damage caused to said transformed plants from feeding insects, comprising applying to said plant an insecticidal chemical and/or biological agent, with the proviso that said biological agent is not a polyhedrosis virus, and subject-matter related thereto.

2. Claims: 1,2,7-10,15-21 partially

idem, wherein the constituent protein is an ER-specific chaperone BiP protein



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Office

**SUPPLEMENTARY
PARTIAL EUROPEAN SEARCH REPORT**

under Rule 46, paragraph 1 of the European Patent Convention
Application Number EP 00 90 8824

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 97 12906 A (COMMW SCIENT IND RES ORG ;DALL DAVID JAMES (AU)) 10 April 1997 (1997-04-10) * the whole document *	1,7-9, 15-18,21	A01H5/00 A01N63/02
A	EP 0 596 508 A (THOMPSON BOYCE PLANT RES) 11 May 1994 (1994-05-11)		
P,X, D	HUKUHARA TOSHIKO ET AL: "Increased baculovirus susceptibility of armyworm larvae feeding on transgenic rice plants expressing an entomopoxvirus gene." NATURE BIOTECHNOLOGY, vol. 17, no. 11, November 1999 (1999-11), pages 1122-1124, XP002231409 ISSN: 1087-0156 * the whole document *	1-8	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
			A01N C07K C12N
LACK OF UNITY OF INVENTION			
The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely			
see sheet B			
The present partial European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims.			
Place of search MUNICH		Date of completion of the search 17 February 2003	Examiner Kania, T
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons 8 : member of the same patent family, corresponding document	

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 00 90 8824

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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17-02-2003

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